

Bridging the Gap between TGF- β /Smad Signalling and Tumorigenesis Arising from *Clonorchis sinensis* Induced Hepatic Fibrosis

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Abstract

Clonorchiasis is a parasitic infection caused by food borne trematode, *Clonorchis sinensis* that is mainly prevalent in Asian countries, including South Korea, China, northern Vietnam, Japan, as well as far-eastern Russia, in which over 35 million people are the casualties. Clonorchiasis is characterized by the development of hepatic fibrosis. Upon chronic liver injury following the *C. sinensis* infection, hepatic fibrosis develops into cholangiocarcinoma with a concomitant genetic and epigenetic mutations. Cholangiocarcinoma represents important clinical manifestation of *C. sinensis* infection and causes high rate of morbidity. TGF- β /Smad signalling is known to initiate hepatic fibrosis following the hepatic injury. However, little is known about the role of TGF- β /Smad signalling during *C. sinensis* induced hepatic injury and the underlying contribution of TGF- β /Smad signalling in the development of cholangiocarcinoma. The expression dynamic of TGF- β /Smad signalling and their role in the development of hepatic fibrosis in *C. sinensis* infected BALB/c mice have been investigated. Concomitantly but irrespective to *C. sinensis* infection, the role of hepatic epithelial TGF- β during hepatic fibrosis and the development of cholangiocarcinoma arising from hepatic epithelial cells have also been dissected. Both findings will be reviewed in this paper. Thereby, the link between TGF- β /Smad signalling, hepatic fibrosis during *C. sinensis* infection, and cholangiocarcinoma could be drawn clearly.

Keywords: *Clonorchis sinensis*, TGF- β /Smad signalling, Hepatic fibrosis, Cholangiocarcinoma

INTRODUCTION

Clonorchiasis is a parasitic infection caused by food borne trematode, *Clonorchis sinensis*. *C. sinensis* infection is mainly prevalent in Asian countries, including South Korea, China, northern Vietnam, Japan, as well as far-eastern Russia, in which over 35 million people are the casualties (Qian, *et al.*, 2016). People become sick after eating raw or insufficiently cooked aquatic product containing metacercariae or after drinking the contaminated water. In the duodenum, the ingested metacercariae will develop into adult worm by the stimulation of gastric juice and migrate to intrahepatic bile duct (Hong, *et al.*, 2012). Following the migration of the adult worms into intrahepatic bile duct, transient hepatic fibrosis is developed at 7 days after infection. Hepatic fibrosis is a wound healing response against hepatic injury by the adult worms and characterized by an excessive

accumulation of extracellular matrix (ECM) component, such as collagen and fibronectin (Wynn, *et al.*, 2012).

Patients normally experience mild symptoms during transient hepatic fibrosis, such as diarrhea and abdominal pain. The moderate amounts of adult worms (101-1000) can cause loss of appetite, rash, edema, night blindness, swollen abdomen, and enlargement of the liver. Eventually, the acute symptoms will abate and the liver architecture is restored to normal following the treatment with praziquantel. However, if the infection persists and reach chronic stage, as in the case of *C. sinensis* infection, in which adult worm could reside in the

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bile duct for 20-40 years, the liver become malfunction. As the consequence of progressive fibrosis, there will be liver obstruction, which could develop into cholangiocarcinoma. Cholangiocarcinoma is a malignant tumor that arises from hepatic bile ducts and represents important clinical manifestation of *C. sinensis* infection. The International Agency for Research on Cancer (IARC) classifies *C. sinensis* as a probable carcinogen (group 2A). Nevertheless, the mechanism of *C. sinensis* carcinogenesis remains elusive (Keiser & Utzinger, 2009; Wu, *et al.*, 2012).

THE ROLE OF CYTOKINE(S) DURING HEPATIC FIBROSIS

The role of cytokine system is highly appreciated to regulate liver fibrosis during *C. sinensis* infection and eventually control the development of cholangiocarcinoma. In fact, many excretory/secretory proteins from *C. sinensis* tegument (ESPs) including secretory phospholipase A (2) (CsPLA2), lysophospholipase (CslysoPLA), fructose-1, 6-bisphosphatase (CsFBPase) and Fe heavy chain protein (CsFHC) have been reported to induce the production of regulatory cytokine TGF- β (Hu, *et al.*, 2009). The TGF- β , especially TGF- β 1 is acknowledged as a critical fibrogenic stimulus. During chronic hepatic injury, inflammatory cells secreting inflammatory mediators, including TGF- β , are recruited in the area of tissue injury. TGF- β enhances the activation of the hepatic stellate cells (HSCs) or pericytes. The HSCs will then differentiate from vitamin A storing cells into myofibroblasts (MFs) expressing alpha smooth muscle actin (α -SMA). The MFs is the primary source of fibrogenic population in the liver and secrete an excessive amount of ECM (Mao, *et al.*, 2015; Yan, *et al.*, 2015). In addition to HSCs activation, TGF- β also mediate trans-differentiation of hepatic epithelial cells (hepatocytes and cholangiocytes) into MFs through biological process known as EMT (epithelial mesenchymal transition). EMT allows a closely attached epithelial cells with apical-basal polarity to migrate and acquire a mesenchymal cell phenotype (migratory capacity, invasiveness, resistance to apoptosis, and production of ECM). EMT has been associated with wide range of biological processes, not only tissue regeneration and fibrosis but also with embryonic development

and cancer progression (Hernandez-Gea and Friedman, 2011).

Transforming growth factor- β (TGF- β) signalling is important in the regulation of cell growth, differentiation, and development in a wide range of biological process (Warmflash, *et al.*, 2012). TGF- β has been implicated in tumour progression, however the function and utility of TGF- β are very complex and highly dependent on the cellular context it is involved with. Of note, TGF- β is reported to exhibit both tumour suppression and promotion in temporally and spatially different level (Padua and Massague, 2009).

In general, TGF- β is secreted by variety of cell types in three major isoforms, namely TGF- β 1, TGF- β 2, and TGF- β 3. The most important fibrogenic stimulus, TGF- β 1, is stored as an inactivated homo- or hetero-dimers of protein bound to a latency-associated protein and activated through proteolytic cleavage by endoproteases. TGF- β mediates signalling through serine/threonine kinase, TGF- β receptor type I (T β RI) and TGF- β receptor type II (T β RII). Initially, TGF- β will bind to T β RII. Thereafter, T β RII will dimerizes with T β RI and bind to Smad2 and Smad 3 transcription factors. The T β R/Smad complex becomes phosphorylated and is released into the cytosol, where it will associate with Smad4. The resulting complex will translocate into the nucleus and regulate gene transcription, including ECMs. The TGF- β /Smad signalling can be inhibited endogenously by Smad6/7, which prevents the binding of Smad2/3 to the receptor. In regulating various biological process, TGF- β also elicits non-canonical signalling response through signalling crosstalk with Ras and MAP kinase, Wnt signalling, and Notch signalling, which adding the complexity of TGF- β signalling (Minoo and Li, 2010; Remi, *et al.*, 2004; Xu, *et al.*, 2009).

Of note, very little is known about the role of TGF- β /Smad signalling during hepatic fibrosis caused by *C. sinensis*. In their recent publication, Yan and co-workers (2015) investigated the expression dynamic of TGF- β /Smad signalling and analysed their possible role in the development of hepatic fibrosis in *C. sinensis* infected BALB/c mice. Concomitantly but irrespective to *C. sinensis* infection, Mu and co-workers (2016) used a T β RII knockout mice to show the role of epithelial TGF- β

during hepatic fibrosis and the development of cholangiocarcinoma arising from hepatic epithelial cells.

The expression dynamics of TGF- β /Smad signalling in the development of hepatic fibrosis caused by *C. sinensis*

The study by Yan and co-workers (2015) highlights the involvement of TGF- β /Smad signalling in the development of hepatic fibrosis caused by *C. sinensis*. The authors used female BALB/c mice as an animal model for developing hepatic fibrosis by per-oral administration of *C. sinensis* metacercariae. Following the infection of mice with *C. sinensis* metacercariae, mice could develop a moderate periductal fibrosis at 4 weeks post infection (p.i). Meanwhile, the massive deposition of collagen was observed from the portal areas to liver lobules after 8 weeks p.i using hematoxylin and eosin as well as Masson's trichrome staining. The development of hepatic fibrosis was further validated by the expression of pro-fibrotic molecular markers during *C. sinensis* infection. The total mRNA was extracted from liver tissue. Using qPCR, the authors showed that the mRNA level of α -SMA and collagen α 1 (Col1a) were significantly increased from 4 weeks p.i to 16 weeks p.i compare to control. In order to investigate the activation of TGF- β /Smad signalling during hepatic fibrosis induced by *C. sinensis* infection, the authors examined the mRNA and protein expression within TGF- β /Smad signalling pathway. As

expected, the hepatic fibrosis mice exhibited high expression of TGF- β 1, Smad2/3, T β RI, and T β RII mRNA. This result implicates the involvement of TGF- β /Smad signalling in hepatic fibrosis following the *C. sinensis* infection (Fig. 1).

Several studies have been conducted and confirmed the role of TGF- β /Smad signalling in parasite-induced hepatic fibrosis. The studies were conducted using various parasites, including the metacercariae of *Echinococcus multicularis*, *Schistosoma japonicum*, and *Schistosoma mansoni*'s soluble egg antigens. The authors generally reported similar finding and pinpointed the role of TGF- β /Smad signalling in the activation of HSCs as the initial step of hepatic fibrosis following the parasitic infection. The expression of TGF- β 1 and Smad2/3 were markedly high in the hepatic areas close to and distant from parasitic lesions, while the expression of Smad7 was essentially down-regulated (Barros, *et al.*, 2014; Chen, *et al.*, 2013; Wang, *et al.*, 2013).

The expression dynamic of Smad proteins was shown by Yan and co-workers (2013). Using qPCR analysis, the mRNA level of Smad4, Smad2, and Smad7 were examined. The expression of Smad4 was increased at 4 weeks p.i, however as the hepatic fibrosis developed, the expression of Smad4 was markedly decreased, suggesting that the effect of Smad4 might occur at early and middle stage of hepatic fibrosis. Nevertheless, the expression of Smad7 was negatively correlated with the expression level of Smad2 during hepatic fibrosis, which further confirmed contribution of TGF- β /Smad signalling.

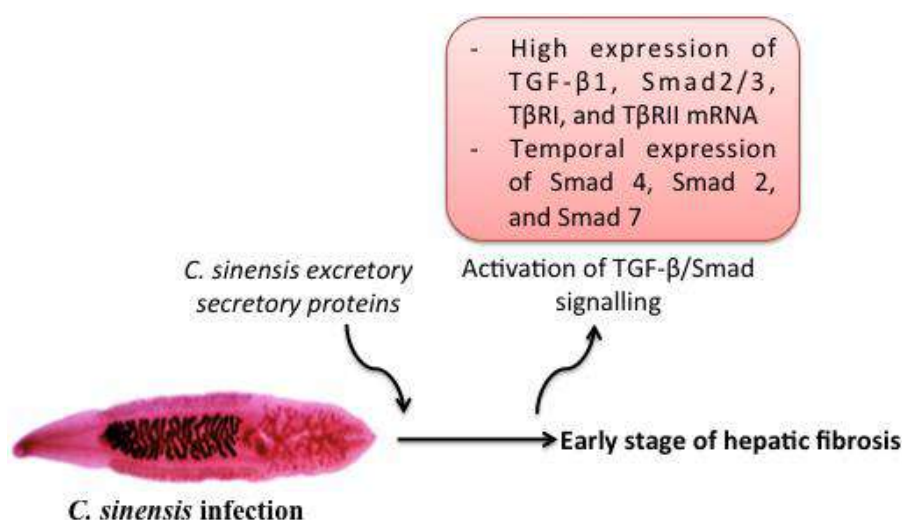


Figure 1. The involvement of TGF- β /Smad signalling during *C. sinensis* infection. The TGF- β /Smad signalling is activated during the early stage of hepatic fibrosis developed after *C. sinensis* infection.

The cellular compartment specific dependent contribution of TGF- β /Smad signalling in hepatic tumorigenesis

In the liver, TGF- β can be produced by infiltrated inflammatory cells and hepatic parenchyma cells, including epithelial cells (hepatocytes and cholangiocytes), endothelial cells, as well as resident non-parenchymal cells (HSCs and Kupffer cells). Moreover, the regulatory output of TGF- β /Smad signalling is known to be temporally and spatially different depending on the cellular context, thereby different cellular compartments of TGF- β could exhibit different effect. Mu and co-workers (2016) attempted to dissect the role of hepatic epithelial TGF- β during hepatic fibrosis and the underlying cholangiocarcinoma.

In order to dissect the role of hepatic epithelial TGF- β , the authors used double transgenic mice expressing floxed T β RII and Albumin-Cre to knockout the TGF- β signalling in the hepatic epithelial (Mu, *et al.*, 2016). They also made use of toxicity-induced fibrosis (carbon tetrachloride/CCl₄ injections in mice) and cholestatic hepatic fibrosis (common bile duct ligation and Mdr2 knockout mice) to develop chronic hepatic fibrosis in mice. Using chronic hepatic mice model only, they showed that TGF- β signalling was activated in both the epithelial and mesenchymal cell compartments of chronically injured liver. Conversely, using double transgenic expressing floxed T β RII and Albumin-Cre in chronically injured mice, they found out that there was no significant difference in the status of hepatic fibrosis compare to control mouse, as shown by the expression of pro-fibrotic molecular markers. However, they showed the markedly elevated expression of keratin. This data suggests that epithelial TGF- β does not contribute to hepatic fibrosis but rather control the expansion of cholangiocytes. Furthermore, TGF- β from other cellular compartments other than hepatic epithelial cells also contribute to parasite-induced hepatic fibrosis, including *C. sinensis* induced hepatic fibrosis.

The authors observed a significant role of T β RII in tumorigenesis caused by the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) loss. PTEN is a tumor suppressor gene that antagonizes and suppresses cell survival as well as cell proliferation (Yin and Shen, 2008). Using triple transgenic mouse where Albumin-Cre drives the loss

of T β RII and PTEN simultaneously in hepatic epithelial cells, they showed that mice deficient of both T β RII and PTEN developed tumours and die at 5-7 months of age, while the PTEN deficient mice were tumour free at the similar age. The tumours were keratin positive and exhibited high expression of cholangiocyte and cholangiocarcinoma markers, suggesting that T β RII and PTEN loss induce cholangiocarcinoma in chronically injured mice.

It was not very clear whether the cholangiocarcinoma developed in mice is hepatocytes or cholangiocytes origin, because the deletion strategy using Albumin-Cre affected both hepatocytes and cholangiocytes. Thereby, to distinguish between TGF- β signalling in hepatocytes and cholangiocytes, the authors used cell type specific ablation strategies to knockout T β RII. In order to develop cholangiocytes specific ablation, they made use of two different mice strains, Prom-1-CreERT2 with floxed T β RII and PTEN as well as K19-CreERT with floxed T β RII and PTEN. Both prominin and keratin 19 are surface markers for cholangiocytes. Prior to conditional deletion, all mice were put in 3,5-diethylcarbonyl-1,4-dihydrocollidine (DDC) to induce cholestatic hepatic injury. Meanwhile, for inducing specific ablation in hepatocytes, the authors used double transgenic T β RII and PTEN floxed mice and AAV8-TBG-Cre virus infection. Interestingly, after being sacrificed, both mice models were shown to develop the characteristics of cholangiocarcinoma irrespective to the cell compartment origin. Moreover, by co-labelling the Cre with GFP, they demonstrated that the developed cholangiocarcinoma in cholangiocytes specific deletion mice, had hepatocytes origin. This result suggests that the loss of T β RII and PTEN in hepatic epithelial cells will give rise to proliferation of cholangiocytes cells derived from hepatocytes cells, which lead to the development of cholangiocarcinoma.

CONCLUDING REMARK

Of note, the contribution of TGF- β /Smad signalling during chronic hepatic fibrosis and *C. sinensis* induced hepatic fibrosis have been validated through the study from Yan and co-workers (2015) as well as Mu and co-workers (2016). During hepatic injury TGF- β proteins secreted from various cells in the liver as well as infiltrated inflammatory cells, inhibit hepatocyte proliferation, induce

hepatocyte apoptosis, and activate HSC to differentiate into MFs. Through canonical Smad signalling, TGF- β proteins regulate gene transcription of ECMs as well as induce the production of pro-fibrosis molecular markers and ECM in MFs. Hepatic fibrosis is developed as the excessive amount of ECM is built up in the liver. However, not all the cells in the liver contribute to the development of hepatic fibrosis following the chronic hepatic injury. Hepatic epithelial TGF- β does not contribute to hepatic fibrosis (Mu, *et al.*, 2016), suggesting the cellular compartment specific dependent contribution of TGF- β /Smad signalling. Accordingly, the identification of major cellular compartment that produces TGF- β /Smad signalling induced hepatic fibrosis is of importance, especially in the context of *C. sinensis* induced hepatic fibrosis. Hepatic fibrosis is a major symptom of Clonorchiasis, which affects 35 million people worldwide. Novel treatment could be established using inhibitor of TGF- β , which target specific cellular compartment in the liver to improve the therapeutic outcome.

The loss of hepatic epithelial T β RII with the accompanying loss of tumour suppressor gene is enough to induce cholangiocarcinoma arising from chronic hepatic fibrosis (Mu, *et al.*, 2016) (Fig. 2). TGF- β has dual role in tumorigenesis, as tumour

suppressor and promoter. However, mutation in tumour suppressor gene may switch tumour suppressor effect of TGF- β into tumour promoter, as shown in the dynamic disposition of hepatic fibrosis into cholangiocarcinoma. In fact, the latest study from Ong and co-workers (2012) support this notion. The whole exome sequencing of eight liver trematode *Opisthorchis viverrini* related cholangiocarcinoma samples showed the somatic mutation in smad4 gene, which concomitant with mutation in tumour suppressor gene *Tp53* and *KRAS* (Ong, *et al.*, 2012). The inactivating mutation of the core element in TGF- β /smad signalling is tumour inherent mechanism to escape from tumour suppressor effect of TGF- β /smad signalling. Thus, allowing tumour cells to acquire proliferative and invasive features.

The future research that focus on the generation of cholangiocarcinoma from *C. sinensis* induced hepatic fibrosis is certainly needed, considering the high mortality rate related to cholangiocarcinoma. The novel findings by Ong and co-workers (2012) as well as Mu and co-workers (2015) should be a starting point for further elucidating the role of TGF- β /smad signalling in the development of cholangiocarcinoma arising from *C. sinensis* induced hepatic fibrosis.

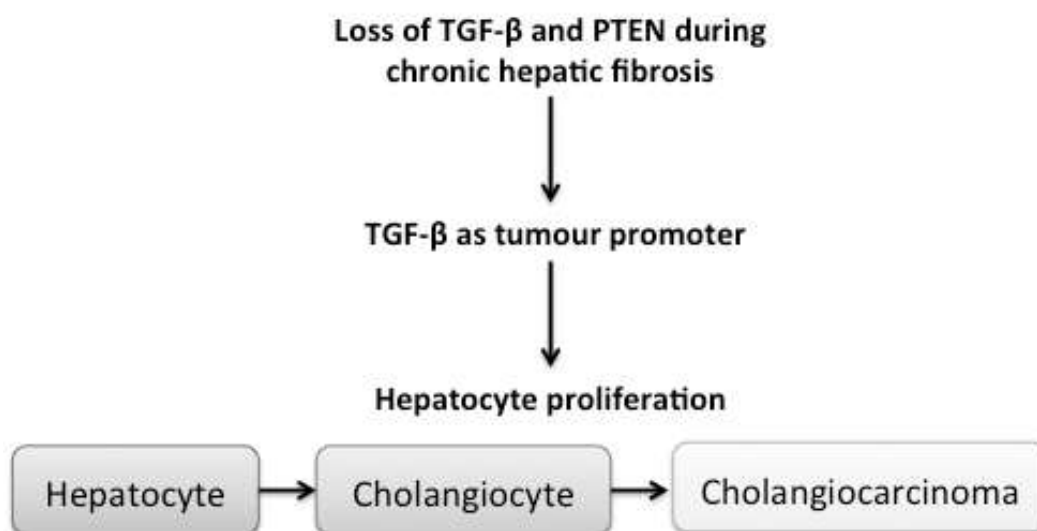


Figure 2. The duality of TGF- β is switched into tumour promoter following the concomitant loss of tumour suppressor gene during chronic hepatic fibrosis.

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